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13. ABSTRACT ***(Maximum 200 Words)***

Sulfur mustard (SM) is a powerful chemical warfare vesicant. No efficient pharmacological antidote is available against this blisterogen. The present study demonstrates the protective effect of newly developed iodine preparation for topical treatment after exposure to SM. In the haired guinea pig model, post-exposure treatment with iodine reduced the degree of skin lesions caused by 1µl SM. We have seen that intervals of 15, 30, and 45 min between exposure and treatment caused statistically significant reduction of 93%, 91% and 50%, respectively, in ulceration area. Longer interval of 60 min also reduced the lesion by 50% but without statistical significance. The longer was iodine left on the skin the better was the protection achieved, whereas the best protection was observed when iodine was left 2 hours on skin. There was also relationship between degree of protection and SM dose. GC-MS studies showed that iodine did not chemically modified SM. We continued to attempt identifying the previously reported protective factor produced in iodine-treated skin by HPLC and HPLC-MS. In the near future intensive effort will be invested in this issue and in further establishment of the protective effect of iodine.

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Ulrich Wegmann August 20, 1999

PI - Signature Date

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INTRODUCTION

Sulfur mustard [Cl-CH_2-CH_2-S-CH_2-CH_2-Cl] is a highly potent alkylating agent and severely cytotoxic vesicant Topical exposure to SM results in erythema which appears within hours of intoxication followed by edema, blistering and ulceration observed within tens of hours following poisoning (1-5) . The devastating effect of SM was demonstrated in several conflicts in this century emphasizing the great need of an efficient pharmacological antidote against mustard gas toxicity. Numerous compounds have been tested for their antidotal activity against SM however, they were too weak to be used as protectants against the vesicant. *The purpose of the present study was to develop a pharmacological antidote for* ***post-exposure*** *treatment of SM-exposed individuals*.

BODY

Background

Our preliminary studies have shown that povidone iodine ointment protects guinea pig skin against SM (6). Gross and histopathological findings showed that application of povidone iodine 20 min or less following exposure to the vesicant resulted in marked protection . The shorter interval between exposure and treatment the better protection was achieved.

However, a weak beneficial effect, if at all, was achieved in cases of longer intervals (more than 20 min) between exposure and povidone iodine treatment. Although 20 min interval may be helpful in emergency of mustard gas attack, we had a strong feeling that

improving the formulation of povidone iodine may contribute to its counterirritating activity. This assumption stemmed from the fact that ***commercial preparations of different sources were highly variable in their antidotal activity***. Moreover, the most successful formulation previously used by us (Fisher Pharmaceuticals, Israel) gave inconsistent and contradicting results in the haired guinea pig model; assuming that some differences in the formulation components may be responsible for the obtained discrepancies in iodine activity. ***Therefore, the first objective of the present study was to develop a formulation that will show the highest protective activity against SM***. This issue is important not only for the soldier in the battlefield but also for our basic science studies aimed to clarify the molecular mechanism of the protective activity of iodine.

Intensive effort was invested in developing the right formulation for addressing our main objectives. We have tested about 30 formulations of povidone-iodine and iodine. The most successful one, containing 2% I_2, was shown to be the most potent in preventing skin lesions caused by SM. In the near future a patent application will be submitted and a copy will be mailed to the USAMRDC.

Experimental procedure

The back of a male guinea pig (700-950 g) was shaved 24 hours prior to the experiment. Anesthesia was achieved by 30 mg/kg pentobarbital ip and maintained (during the experiment) by 15 mg/kg of this agent. From numerous experiments we concluded that the best way to perform the study is a) to apply the vesicant on no more than 6 sites per animal; b) to test only one dose and no more than one time interval between exposure and treatment per animal. Thus, the basic procedure was to apply the vesicant on 6 sites

of animal back while 3 of them were treated with iodine and the others were the controls (untreated). Since our formulation is in a liquid form which should stay on the skin long enough after exposure, a well was constructed by the following procedure. A cylindrical well, open at both ends, was cut from a plastic tube and was applied a thin layer of commercial silicon sealing ointment to one edge of the well. The well was then attached to the animal's back. The skin area covered by the well was 1.8 sq. cm. Up to three wells were constructed on the back of each guinea pig. The irritant was applied on the center of the preconstructed well and after certain time intervals 1 ml iodine was added. For control, each experiment included animals that were exposed to SM only without iodine treatment. Two hours later (otherwise indicated), the iodine and well were removed and the animals were daily checked for gross pathology. In the end of experiment skin samples were removed for histology.

RESULTS

Establishment of the protective effect of iodine

Protective effect of iodine against 1µl SM

Exposure of guinea pig skin to 1µl (1.2 mg) net irritant followed by iodine treatment applied 30 min after the vesicant resulted in significant reduction in skin lesions as compared to the control sites that were exposed to SM only-without iodine treatment (see Fig. 1a). ***It is clearly demonstrated that iodine protects against SM at interval of 30 min between exposure and treatment***.

Moreover, ***longer intervals such as 45 and 60 min between SM exposure and iodine treatment gave also beneficial results*** (Figures 1b, 1c). Both photos show that even at 45 min (left photo) and 60 min (right photo) interval between exposure and treatment iodine is still effective as a counterirritant. In both animals the 3 right applications were treated with iodine while the three left ones were controls (SM only). In the left animal (45 min interval) the two lower right applications show marked smaller ulceration area as compared to their controls (without iodine). Similar findings were obtained with 60 min interval, namely, the ulceration areas of the two upper right sided sites were smaller than those of their controls (left sides).

Quantification of the protective effect of iodine

In order to quantify the protective effect of iodine against SM in the haired guinea pig model, we measured the ulceration area of each application and summarized the results at each time interval between exposure and treatment (Fig. 2). Erythema could not be quantified because of masking effect by iodine staining.

It is clearly demonstrated ***that iodine protected the guinea pig skin against net SM at time intervals of 15 and 30 min between exposure and treatment***; the mean ulceration area was 6.6% and 9.2% of the control (SM only without iodine), respectively. ***The low p values for statistical significance are speaking for themselves***. Moreover, ***the iodine protective effect was also demonstrated at longer time interval such as 45 min*** in which the mean affected areas were 50% of the control (SM only). However, at 60 min interval between exposure and iodine treatment, although not statistically significant, there was a clear trend of reduction in the ulceration area. Interestingly, in spite of the high SD (standard deviation) values (due to the high variability in the results-a typical

phenomenon in humans as well), there was statistical significant difference between iodine-treated and the control group even at long interval such as 45 min.

Adjacent effect of iodine

During our experiments we had the impression that iodine may influence its surrounding area (not treated with iodine) and may have adjacent beneficial effect on other sites exposed to SM only. In order to solve this problem, we compared the size of lesions of controls (SM only) of animals treated with iodine to those of animals devoid of iodine treatment (real controls) (Fig. 3). It can be shown that there is a trend of the "real controls" to have larger ulceration size than the controls of animals that were exposed elsewhere to iodine. Although not statistically significant, ***this trend may indicate for adjacent activity of iodine, namely, some protective influence on the surrounding area as well***.

How long should iodine be left on the skin?

The above mentioned studies were carried out with iodine left on the skin for 2 hours. In order to test whether shorter periods are suitable for protection against SM, guinea pigs were exposed to 1 µl SM and, 30 min later, they were treated with iodine left on the skin for 30 and 60 min. Although the area of ulceration was reduced to 86% and 66% of the control (without iodine), respectively, results were not statistically significant different from the control. Thus, ***iodine should be left on the skin for at least 2 hours***. Longer periods are currently tested.

Protective effect of iodine against 2µl SM

In order to test whether iodine-induced protection is dependent on SM quantity, a doubled dose was tested, namely, guinea pigs were exposed to 2µl (2.4 mg) SM and, after certain time intervals, were treated with iodine (left on the skin for 2 hours). Recent but not yet conclusive results showed that intervals of 15 and 30 min between exposure and treatment the ulceration area was 12% and 47% of the control, respectively. At that stage of study, longer intervals (i.e. 45 and 60 min) did not show marked beneficial effect of iodine. It is planned in the near future to complete this part of study in order to support us with final conclusions.

Since the present iodine formulation was shown as an efficient protectant, low SM doses such as 0.5, 0.2 and 0.1 mg/sq. cm were not tested because the most relevant doses in emergency are the high SM doses (1, and 2µl) tested by us.

Effect of another oxidizer: In order to verify whether other different oxidizing agent may also protect the skin against SM we tested silver sulfadiazine, a widely used agent for topical treatment of skin infections, while the silver atom has oxidizing properties. This agent has some beneficial effect against SM (data not shown), however, to a much lesser extent than iodine. It might be that the insolubility of this agent may contribute to its relative inefficiency.

Verifying the molecular mechanism of the protective activity of iodine

Is SM chemically inactivated by iodine?

In order to answer this question SM (1µl) was incubated (at room temperature) with 10µl 2% iodine preparation (the formulation used for the previously described guinea pig studies) for 60 min. The mixture was extracted with 1 ml methylene chloride and 1µl of the extract was injected into GC/MS using electron impact detector for analysis. For comparison, the same procedure was carried out with iodine-free preparation (control). GC/MS analysis showed that ***SM was not chemically modified by iodine*** (Fig. 4). Furthermore, quantitative measurements showed higher SM levels in the iodine-containing tubes than in the controls (Table 1). This phenomenon may stem from the low pH of iodine preparation (iodine dissolution causes acidification by HI liberation), resulting in decreased rate of hydrolysis of the vesicant. These findings are in agreement with our previously published data showing that monofunctional mustard is not chemically affected by iodine (6).

In this view, it appears that a pharmacological mechanism rather than chemical inactivation is involved in the iodine-induced protection. This assumption is supported by our previous findings ***demonstrating the protective effect of iodine against thermal burns***. We have shown that topical treatment with povidone iodine immediately after exposure to heat reduced and many times prevented skin damage in humans (7). Taking together, it appears that iodine is operating by influencing dermal events.

Mechanism of the protective activity of iodine

Our recent studies indicated that the neuromodulator substance P might be involved in evolution of skin damage caused by exposure to heat or chemical irritant. We have shown that substance P is released from skin terminals upon exposure to high temperature and exposure to the alkylating agent nitrogen mustard (8). It was assumed that substance P is involved in the initiation of the inflammatory process, leading to the erythema, edema and finally to blister and ulceration. Our initial way of thinking was that iodine oxidizes the released substance P by conversion of its methionine at the 11^{th} position to its less active sulfoxide (or sulfone) forms, thus, preventing the activity of substance P and protecting the skin against the noxious stimuli.

In order to test this hypothesis we have examined whether the oxidation products of substance P are produced due to iodine treatment. Our approach was based on the fact that sulfoxide, sulfone and the parent peptide substance P can be separated by HPLC and all of them are recognized by anti substance P antibodies used for radioimmunoassay (RIA). Thus, the oxidation products were identified by extraction of the skin, separation by HPLC and testing each of the eluted fractions by RIA.

We realized that the sulfoxide form was detected after exposure to the alkylator only (without iodine treatment), indicating that the oxidized substance P plays no role in iodine-induced protection. However, in case the exposed skin was treated with iodine, ***a new compound (elution at 25 min) was detected***. This immunoreactive activity did not appear after stimulus only or in a control skin (without exposure and treatment).

Further studies using large amount of skin revealed that this 25 min fraction has protective effect against skin lesions induced by both chemical and thermal stimuli.

Intradermal injection of the HPLC fraction (diluted appropriately with saline) followed

by exposure to stimuli caused marked protection in comparison to the control that received injection of the vehicle.

The most important issue is to chemically identify the 25 min fraction (Fig. 5). Mass spectra (MS) analysis (for peptides) did not support us with positive results (no peptide was detected). This may stem from the following reasons: a) low quantities of the factor; b) impurities that interfere with the analysis; c) the factor is not a peptide.

It is planned to a) obtain large amounts of the factor by using preparative (instead of analytical) HPLC column; b) further purify the factor by using different elution programs and elution solvents. C) employ another type of MS analysis (thermospray, FAB, DCI) in case of non-peptide compound.

Another approach to cope with the chemical identification of factor(s) involved in the iodine-induced protection process included HPLC-MS thermospray analysis for non-peptide compounds. We showed that iodine-treated skin extract had five peaks (Fig. 6) that did not appear in the control skin extract. The detected molecular weights were 283, 327, 371, 415 and 459. It is planned a) to obtain fraction(s) of interest by testing for protective activity against SM (intradermal injections-as previously described); b) to obtain large amounts of the fraction(s) for MS chemical identification; c) to synthesize of the factor(s) and to test its (their) pharmacological and toxicological properties.

Discussion

The present study demonstrates the protective effect of iodine against SM in the haired guinea pig animal model. The recently developed iodine formulation was shown to be effective even at intervals of 45 and 60 min between exposure to 1μl SM and iodine

treatment. As expected, the shorter interval between exposure and treatment the more protection was achieved. The time that iodine should be left on the skin was shown to be detrimental in iodine-induced protection. Interestingly, the adjacent effect of iodine, namely its influence on adjacent, non treated areas, indicates for either iodine diffusion into adjacent, or production of a factor that protects their close environment against the vesicant. Identification of this factor is on of the main roles of future research plan. The relevance to the original hypothesis, namely, that iodine protects the skin against SM, is obvious.

KEY RESEARCH ACCOMPLISHMENTS

The present findings address the following SOW missions (modifications of our letter of April 29, 1999) approved by the Grant Officer (July 3, 1999):

- Iodine formulation was developed
- There was relationship between degree of protection and a) time interval between SM exposure and iodine treatment, b) SM dose (to be completed in neat future).
- There was relationship between degree of protection and time that iodine was left on the skin.
- Chemical analysis of SM after reaction with iodine was performed.
- The protective factor(s) was (were) further characterized.

REPORTABLE OUTCOMES

- Presentation at the European Society of Toxicology, Oslo, June 1999.
- Wormser U, Nyska A, Brodsky B, (1999) Iodine protects skin against chemical and thermal burns. Toxicol. Lett. Suppl. 1/99, P311.

- Papers on the protective effect of iodine and on the effect of oxidizers on SM are under preparation.
- All data described in this document will be presented in the SOT 2000.
- A patent application on iodine formulation will be submitted in the near future.

CONCLUSIONS

1. Iodine protects the guinea pig skin against SM.
2. The degree of iodine-induced protection is dependent on time interval between exposure and treatment; at time intervals of 15, 30 and 45 min the degree of protection was 93%, 91% and 50%, respectively.
3. The degree of iodine-induced protection is dependent on how long iodine is left on the skin and on SM dose.
4. Iodine also affects the surrounding area of application (adjacent effect).
5. SM is not chemically inactivated by iodine.

"**So what**": The present results offer an efficient topical preparation for post exposure treatment in case of SM exposure of soldiers and civilians in emergency. The study may lead to better understanding of the molecular mechanism of SM activity and to clarification of iodine induced protection.

REFERENCES

1. J.C. Dacre, M. Goldman, *Pharmacol.Rev.* **48**, 289 (1996).
2. S.G. Mellor, P. Rice, G.J. Cooper, *Br.J.Plast.Surg.* **44**, 434 (1991).

3. W.J. Smith, M.A. Dunn, *Arch.Dermatol.* **127**, 1207 (1991).

4. A.P. Watson, G.D. Griffin, *Environ.Health Perspect.* **98**, 259 (1992).

5. U. Wormser, *Trends.Pharmacol.Sci.* **12**, 164 (1991).

6. U. Wormser, B. Brodsky, B.S. Green, Y.R. Arad, A. Nyska, *Arch.Toxicol.* **71**, 165 (1997).

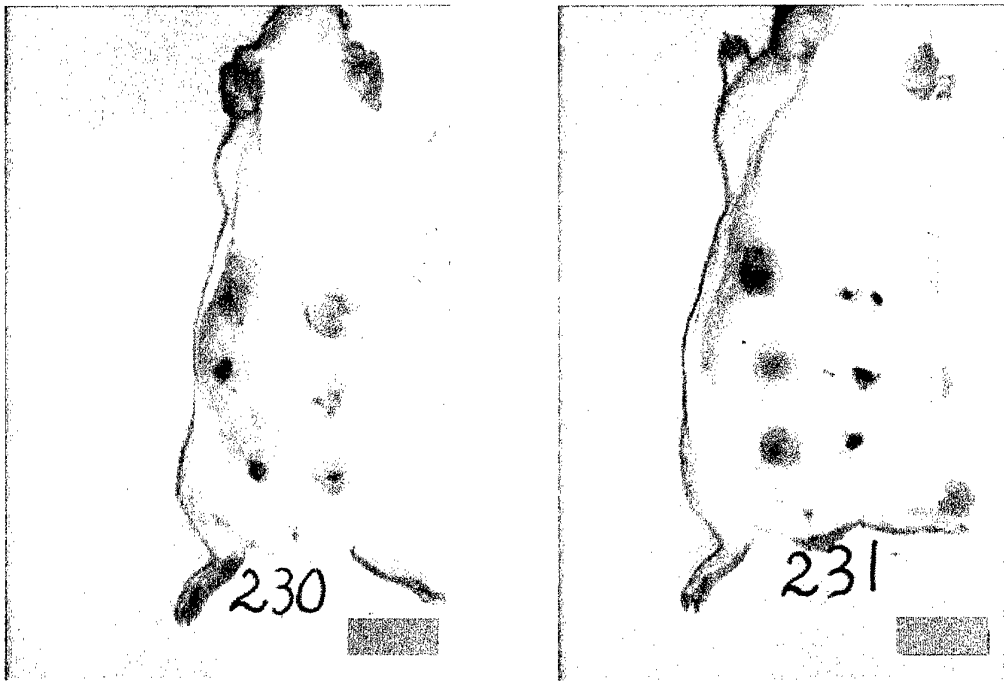
7. U. Wormser, *Burns.* **24**, 383 (1998).

8. U. Wormser, S. Shoham, Brodsky B., *Skin Pharmacol.Appl.Skin Toxicol.* **12**, 98 (1999).

APPENDICES

Fig. 1: Illustration of the protective effect of iodine against SM.

Fig. 1a



One microliter of net irritant was applied on each application site. Iodine was applied 30 min after the vesicant. As can be shown, 3 right sites (left photo) and 3 left sites of exposure (right photo) were treated with iodine while the rest are controls (SM only). Photos were taken 2 days after treatment.

Fig. 1b

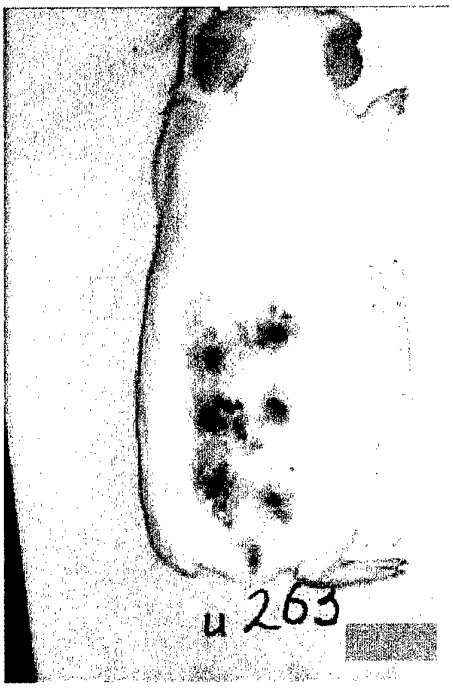
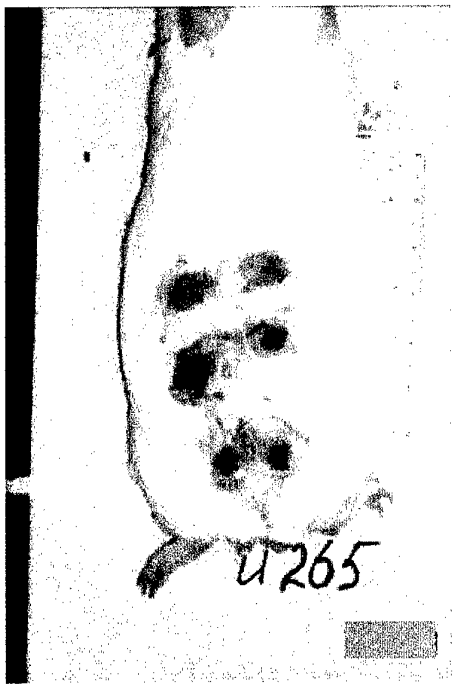
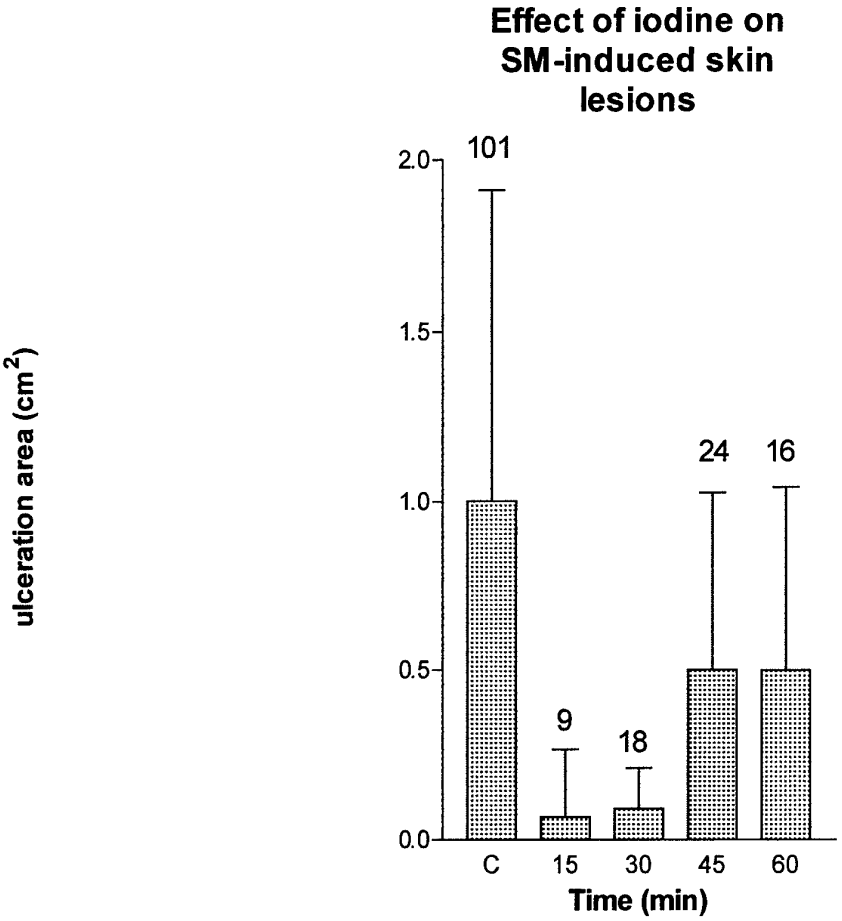


Fig. 1c



One microliter of net irritant was applied on each application site. Iodine was applied 45 min (left photo) and 60 min (right photo) after the vesicant. In both animals the 3 right applications were treated with iodine while the three left ones were controls (SM only). In the left animal (45 min interval) the two lower right applications show marked smaller ulceration area as compared to their controls (without iodine). Similar findings were obtained with 60 min interval, namely, the ulceration areas of the two upper right sided sites were smaller than those of their controls (left sides).

Fig. 2



Guinea pig skin was exposed to 1µl net SM and treated with iodine at the indicated time intervals after exposure. C indicates SM only without iodine treatment. The ulceration area was measured by means of gross pathology evaluation. Numbers on bars indicate numbers of applications (n) at each time interval. Results are expressed as mean ±SD using one way ANOVA and the Dunnett's test (post test) for comparison between the control and each of the other groups.

$p<0.0001$ (ANOVA test)

Comparison between the iodine-treated groups and the control (Dunnett) revealed the following results:

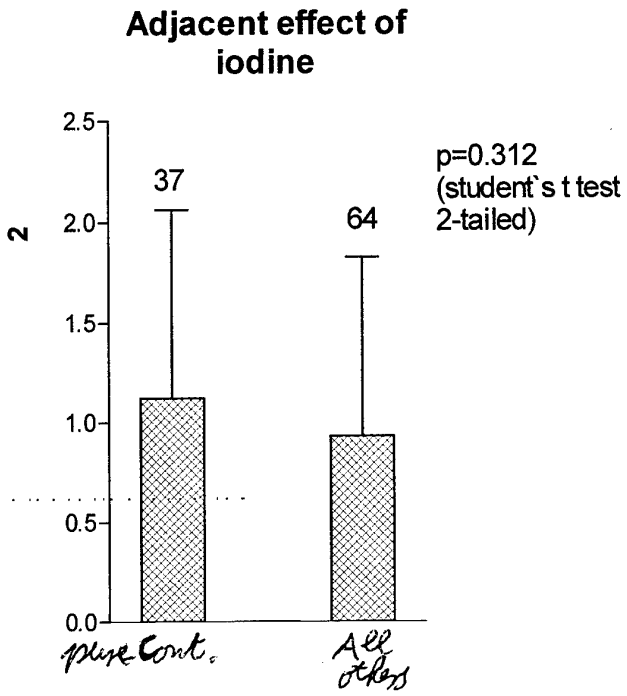
Control versus 15 min interval $p<0.01$

Control versus 30 min interval $p<0.01$

Control versus 45 min interval $p<0.05$

Control versus 60 min interval $p>0.05$

Fig. 3



The ulceration area after exposure to SM (only) was evaluated. There were guinea pigs that were exposed to SM only-and were not treated with iodine at all; these are "pure cont". "All others" means ulceration area after exposure to SM only, but, on the same guinea pig there were also other exposure sites that were treated with iodine (at all time intervals between exposure and treatment i.e. 15, 30, 45, 60 min).
Note that there is no statistical difference between the groups, however, there is a trend of "all others" to be smaller than the real controls, indicating for adjacent protective activity of iodine.
Numbers on bars indicate number of applications (n).

Fig. 4

Typical GC-MS profiles of SM.

SM (1µl) was incubated (at room temperature) with 10µl 2% iodine preparation for 60 min (left profile). As a control, the same procedure was carried out with iodine-free formulation (right profile). The mixture was extracted with 1 ml methylene chloride and 1µl of the extract was injected into GC/MS using RPX1 column (30m) and electron impact detector for analysis. Running conditions were as follows: start at 90°C, then temperature gradient of 10°C/min for 7 min (reaching 160°C), then additional 3 min at 160°C.

Each run is composed of the upper part representing the GC separation profile showing the SM peak and the lower part representing its MS analysis. Note that the additional small peak (right to SM) is a component of iodine formulation.

This analysis shows that SM is not chemically modified by iodine under the specific experimental conditions described above. On the contrary, the amount of SM in the presence of iodine was higher than that of the control (as determined by peak area). Note that the ordinate scale of the left profile (iodine-treated) is smaller than that of the right one (control).

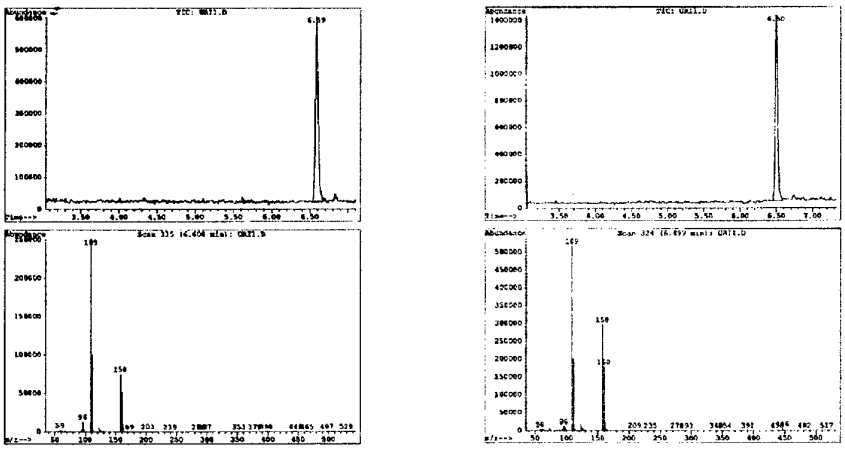


Table 1: Effect of iodine on SM: quantitative determination.

Procedure	SM (µg(±SD
a) SM + I_2 for 60 min	0.42±0.06
b) SM + vehicle for 60 min	0.21±0.03
c) SM + vehicle and immediate extract	1.2±0.23

The experimental details are described in legend to Fig. 4. Procedure c means that SM was added to the vehicle and the mixture was immediately extracted with methylene chloride. SM was quantified by integration of the peak area of the GC profile using appropriate standard. Each data is the mean ± SD of 3 independent measurements.

It can be shown that only 17.5% of the SM left after incubation with iodine-free formulation for 60 min (due to hydrolysis) whereas 35% was left in the presence of iodine. The reduced rate of hydrolysis in the presence of iodine is due to the low pH of the iodine-containing preparation whereas the iodine-free formulation has neutral pH in which increased this process.

Fig. 5: HPLC profile of skin extract.

Guinea pig (after exposure) was treated with iodine for 2 hours and the extract was injected to HPLC. The peak eluted at 25.4 min (marked with arrow) did not appear in the control (exposure without iodine).

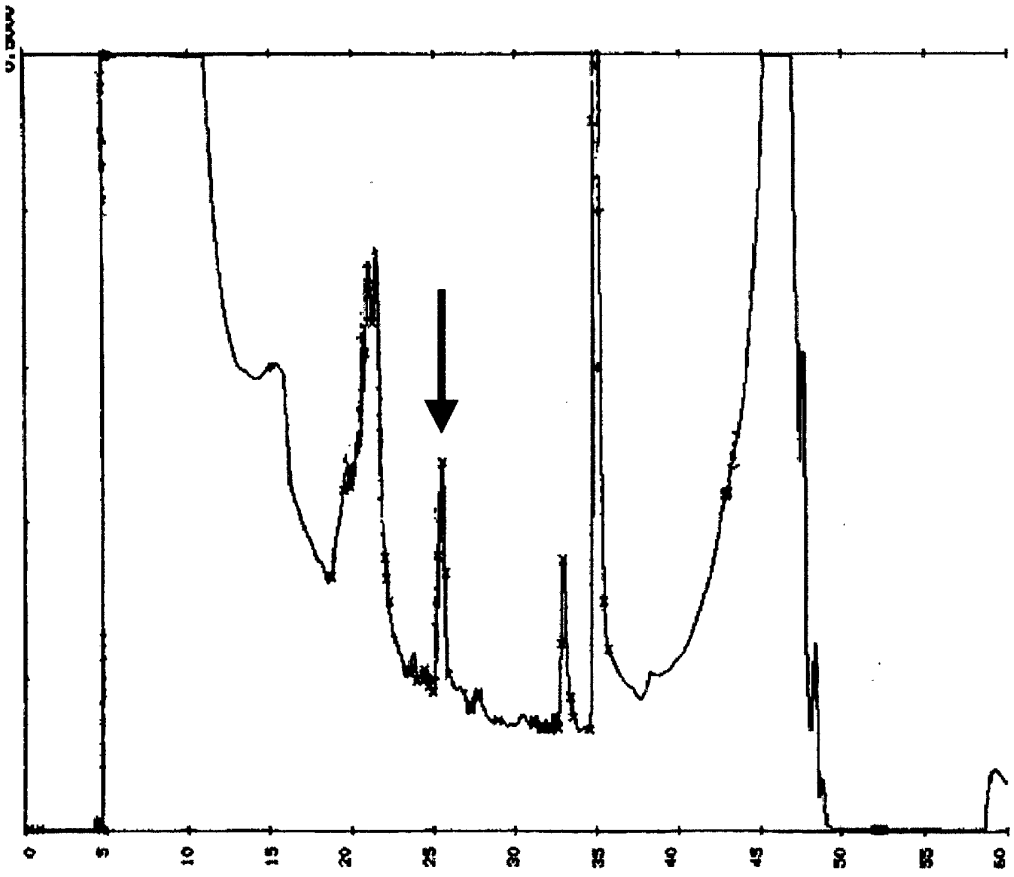
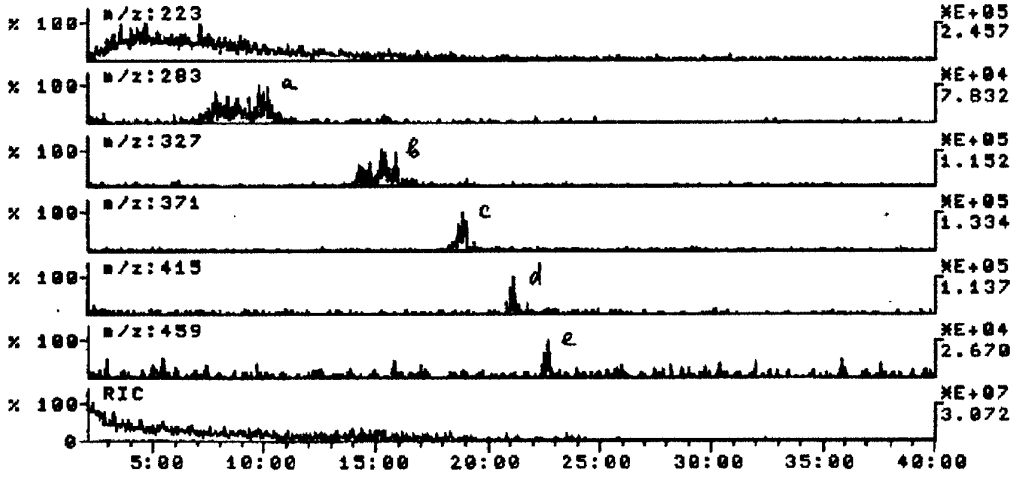
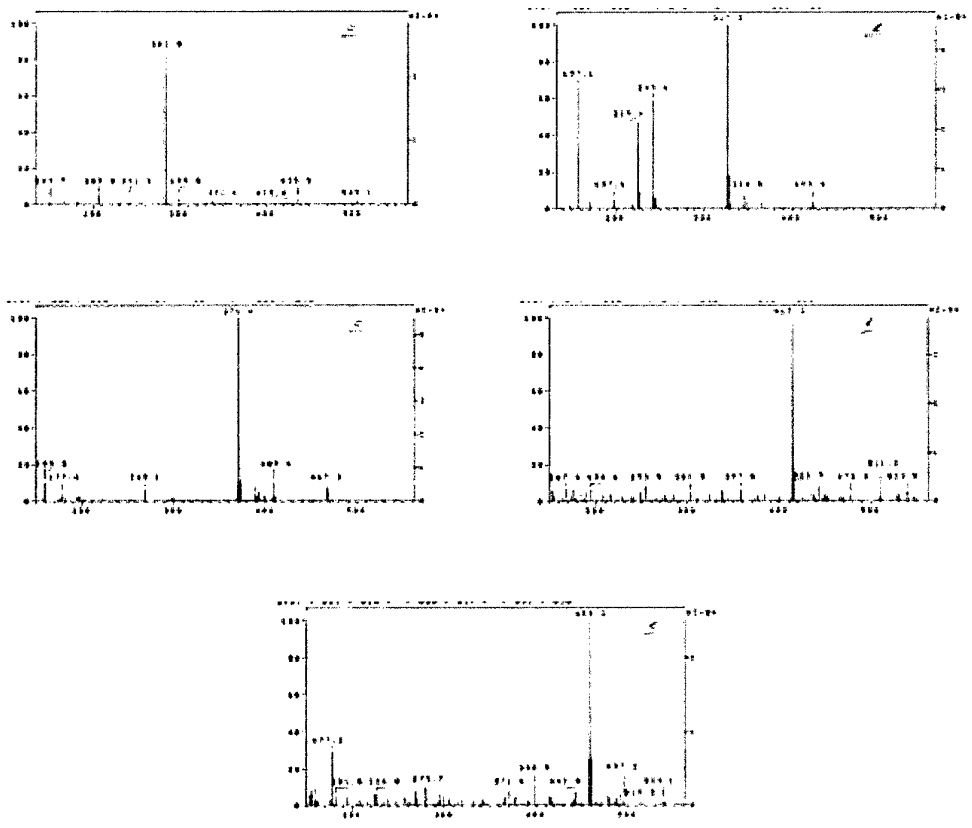


Fig. 6: HPLC-MS thermospray analysis of iodine-treated skin extract; the HPLC elution profile (upper part) with five peaks and their MS spectra (lower part).





PROTECTIVE EFFECT OF IODINE AGAINST SM-INDUCED SKIN LESIONS

Addendum to the annual report

Cooperative Agreement No. DAMD17-98-2-8009

Submitted by

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Introduction

Sulfur mustard [Cl-CH_2-CH_2-S-CH_2-CH_2-Cl] is a highly potent alkylating agent and severely cytotoxic vesicant. Topical exposure to SM results in erythema which appears within hours of intoxication followed by edema, blistering and ulceration observed within tens of hours following poisoning. The devastating effect of SM was demonstrated in several conflicts in this century emphasizing the great need of an efficient pharmacological antidote against mustard gas toxicity. Numerous compounds have been tested for their antidotal activity against SM, however, they were too weak to be used as protectants against the vesicant. *The purpose of the present study was to develop a pharmacological antidote for **post-exposure** treatment of SM-exposed individuals.*
In the present research shaved back of guinea pig was exposed to SM and after certain time intervals the exposed area was applied with iodine. Skin samples were removed after the indicated days for histopathological evaluation.

Microscopic evaluation

Each sample was graded blindly for histopathological changes.The reactive and inflammatory changes were assigned severity grade of 0-4 representing unremarkable, minimal, mild, moderate and marked changes respectively.

Examples of evaluated parameters included: epidermal microblister formation,

ulceration, necrosis, crust formation, hyperkerathosis, acanthosis, edema, hemorrhage, inflammation (acute or subacute, epidermal and/or dermal), presence of giant cells, vascular inflammation and fibrosis.

Histological parameters used to define the grade of "area of epidermal acanthosis"

Grade 6 - Diffuse acanthosis (whole epidermal area is acanthotic).

Grade 5 – About or more than 2/3 of the epidermal area is acanthotic. The rest of the epidermal area is necrotic.

Grade 4 - Less than 2/3 (but more than ½) of the epidermal area is acanthotic. The rest of the epidermal area is necrotic.

Grade 3 - About ½ of the epidermal area is acanthotic. The rest of the epidermal area is necrotic.

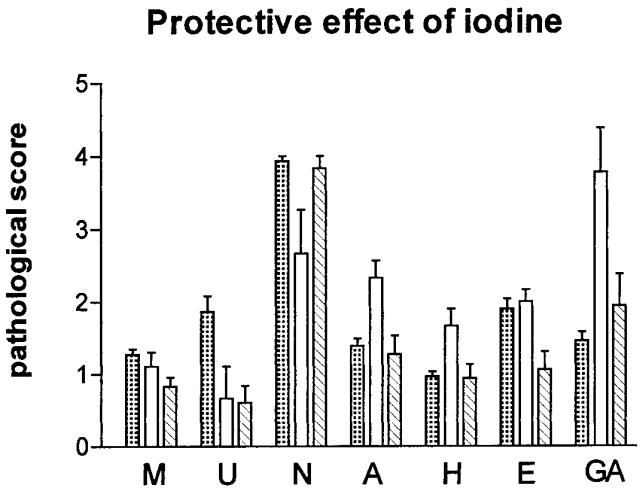
Grade 2 - About 1/3 of the epidermal area is acanthotic. The rest of the epidermal area is necrotic.

Grade 1 - Less than 1/3 of the epidermal area is acanthotic. The rest of the epidermal area is necrotic.

Grade 0 – No acanthosis.

RESULTS

15 and 30 min interval between exposure and treatment-sacrifice after 2 days



Back of guinea pigs (males) was shaved 24 hours prior the experiment. Six sites were exposed to 1µl SM. At certain time intervals after exposure, 3 sites were treated with iodine. In the end of experiment skin was removed for histopathological evaluation.

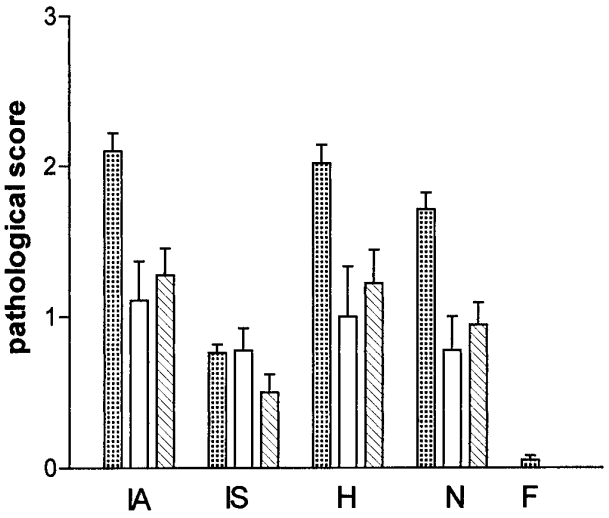
Fig. 1: Histopathological evaluation of the ***epidermal*** layer following exposure to SM and treatment with iodine.

Seven parameters were determined: microvesicles (M), ulceration (U), necrosis (N), acanthosis (A), hyperkeratosis (H), encrustation (E), grade of area of epidermal acanthosis (GA). Each parameter was determined in the following types of treatment: skin exposed to SM only (doted bars), skin exposed to SM followed by iodine treatment 15 min (open bars) or 30 min (hatched bars) later. Animals were sacrificed 2 days after treatment. Results are expressed as mean±SEM using Kruskal Wallis test and Dunnett's multiple comparison post test for statistical evaluation of the difference between control (SM only) (n=59) and 15 (n=9) or 30 (n=18) min interval between exposure and treatment.

Table 1: Statistical evaluation expressed as p values of differences in ***epidermal*** changes between control and 15 and 30 min intervals between exposure and treatment.

Lesion	M	U	N	A	H	E	GA
Cont vs.15 min int.	p>0.05	p>0.05	p>0.05	P<0.01	P<0.01	p>0.05	P<0.001
Cont. vs. 30 min int.	p<0.05	P<0.05	p>0.05	p>0.05	p>0.05	P<0.01	p>0.05
15 vs. 30 min int.	p>0.05	P>0.05	p>0.05	P<0.01	P<0.05	P>0.05	P<0.05

Fig. 2: Histopathological evaluation of the ***dermal*** layer following exposure to SM and treatment with iodine.



Five parameters were determined: inflammation acute (IA), inflammation subacute (IS), hemorrhage (H), necrosis (N), fibrosis (F). Each parameter was determined in the following types of treatment: skin exposed to SM only (doted bars), skin exposed to SM followed by iodine treatment 15 min (open bars) and 30 min (hatched bars) later. Animals were sacrificed 2 days after treatment. Results are expressed as mean±SEM using Kruskal Wallis test and Dunnett's multiple comparison post test for statistical evaluation of the difference between control (SM only) (n=59) and 15 (n=9) or 30 (n=18) min interval between exposure and treatment.

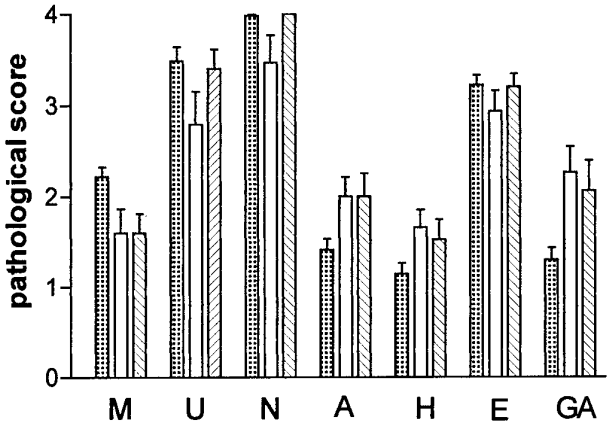
Table 2: Statistical evaluation expressed as p values of differences in ***dermal*** changes between control and 15 and 30 min intervals between exposure and treatment.

Lesion	IA	IS	H	N	F
Cont vs.15 min int.	P<0.01	p>0.05	P<0.01	P<0.01	P>0.05
Cont. vs. 30 min int.	p<0.001	P>0.05	P<0.01	P<0.001	p>0.05
15 min vs. 30 min int.	p>0.05	P>0.05	p>0.05	P>0.05	P>0.05

45 and 60 min interval between exposure and treatment-sacrifice after 5-7 days

Back of guinea pigs (males) was shaved 24 hours prior the experiment. Six sites were exposed to 1µl SM. At certain time intervals after exposure, 3 sites were treated with iodine. In the end of experiment skin was removed for histopathological evaluation.

Fig. 3: Histopathological evaluation of the ***epidermal*** layer following exposure to SM and treatment with iodine.

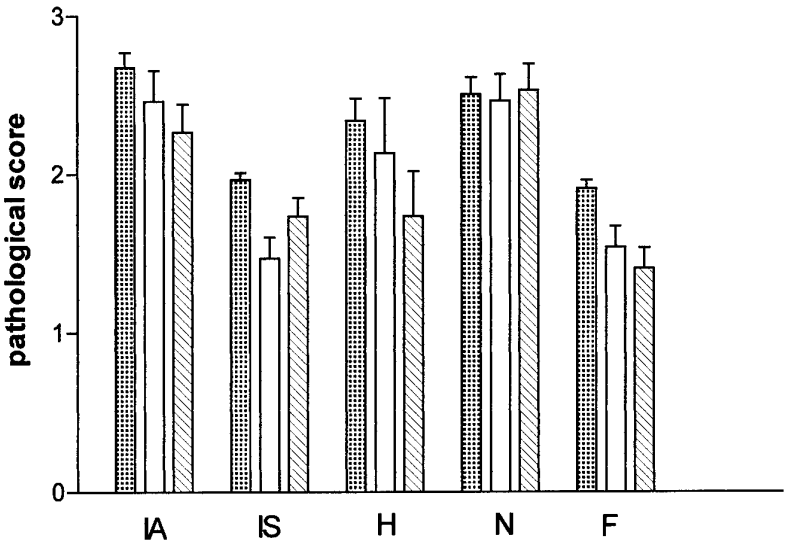


Seven parameters were determined: microvesicles (M), ulceration (U), necrosis (N), acanthosis (A), hyperkeratosis (H), encrustation (E), grade of area of epidermal acanthosis GA). Each parameter was determined in the following types of treatment: skin exposed to SM only (doted bars), skin exposed to SM followed by iodine treatment 45 min (open bars) or 60 min (hatched bars) later. Animals were sacrificed 5-7 days after treatment. Results are expressed as mean±SEM using Kruskal Wallis test and Dunnett's multiple comparison post test for statistical evaluation of the difference between control (SM only) (n=53) and 45 (n=15) or 60 (n=15) min interval between exposure and treatment.

Table 3: Statistical evaluation expressed as p values of differences in ***epidermal*** changes between control and 45 and 60 min intervals between exposure and treatment.

Lesion	M	U	N	A	H	E	GA
Cont vs.45 min int.	P<0.05	P<0.05	p>0.05	P<0.05	P>0.05	P>0.05	P<0.001
Cont. vs. 60 min int.	p<0.05	P>0.05	p>0.05	p>0.05	p>0.05	p>0.05	P<0.05
45 vs. 60 min int.	p>0.05	P>0.05	p>0.05	P>0.05	P>0.05	P>0.05	P>0.05

Fig. 4: Histopathological evaluation of the ***dermal*** layer following exposure to SM and treatment with iodine.



Five parameters were determined: inflammation acute (IA), inflammation subacute (IS), hemorrhage (H), necrosis (N), fibrosis (F). Each parameter was determined in the following types of treatment: skin exposed to SM only (doted bars), skin exposed to SM followed by iodine treatment 45 min (open bars) and 60 min (hatched bars) later. Animals were sacrificed 5-7 days after treatment. Results are expressed as mean±SEM using Kruskal Wallis test and Dunnett's multiple comparison post test for statistical evaluation of the difference between control (SM only) (n=53) and 45 (n=15) or 60 (n=15) min interval between exposure and treatment.

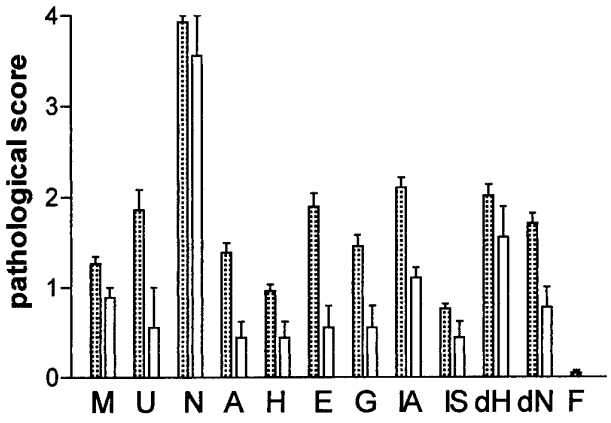
Table 4: Statistical evaluation expressed as p values of differences in ***dermal*** changes between control and 45 and 60 min intervals between exposure and treatment.

Lesion	IA	IS	H	N	F
Cont vs.45 min int.	P>0.05	P<0.01	P>0.05	P>0.05	P<0.05
Cont. vs. 60 min int.	p>0.05	P>0.05	P>0.05	P>0.05	P<0.01
45 min vs. 60 min int.	p>0.05	P>0.05	p>0.05	P>0.05	P>0.05

45 min interval between exposure and treatment-sacrifice after 2 days

Back of guinea pigs (males) was shaved 24 hours prior the experiment. Six sites were exposed to 1µl SM. At 45min interval after exposure, 3 sites were treated with iodine. In the end of experiment skin was removed for histopathological evaluation.

Fig. 5: Histopathological evaluation of the epidermal and dermal layers following exposure to SM and treatment with iodine.

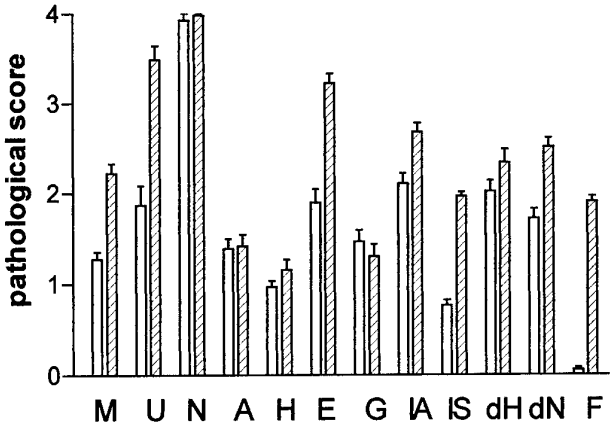


Twelve parameters were determined: epidermal microvesicles (M), epidermal ulceration (U), epidermal necrosis (N), epidermal acanthosis (A), epidermal hyperkeratosis (H), epidermal encrustation (E), grade of area of epidermal acanthosis (G), dermal inflammation acute (IA), dermal inflammation subacute (IS), dermal hemorrhage (dH), dermal necrosis (dN), dermal fibrosis (F). Each parameter was determined in the following types of treatment: skin exposed to SM only (doted bars) and skin exposed to SM followed by iodine treatment 45 min later (open bars). Animals were sacrificed 2 days after treatment. Results are expressed as mean±SEM using the Mann Whitney test for statistical evaluation of the difference between control (SM only) (n=59) and 45 min interval between exposure and treatment (n=9).

Table 5: Statistical evaluation expressed as p values of differences in epidermal and dermal changes between control and 45min interval between exposure and treatment.

Lesion	M	U	N	A	H	E	G
Cont vs.45 min int.	p>0.05	p>0.05	p>0.05	P<0.005	P<0.05	P<0.002	P<0.01
Lesion	IA	IS	dH	dN	F		
Cont vs. 45 min int.	P<0.005	p>0.05	P>0.05	P<0.005	Not applicable		

Fig. 6: Epidermal and dermal lesions induced by SM: histopathological evaluation at different time intervals after exposure to the irritant only.



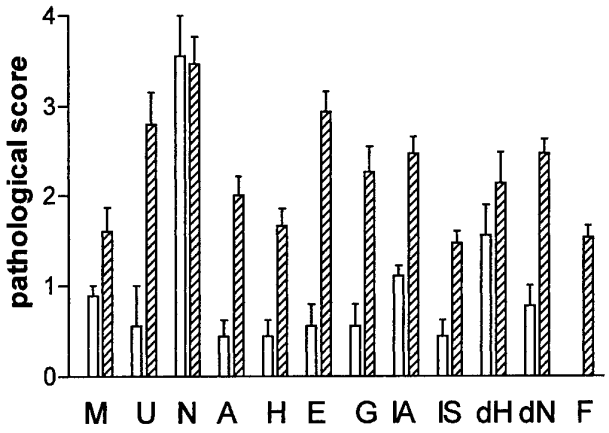
Twelve parameters were determined: epidermal microvesicles (M), epidermal ulceration (U), epidermal necrosis (N), epidermal acanthosis (A), epidermal hyperkeratosis (H), epidermal encrustation (E), grade of area of epidermal acanthosis (G), dermal inflammation acute (IA), dermal inflammation subacute (IS), dermal hemorrhage (dH), dermal necrosis (dN), dermal fibrosis (F). Animals were sacrificed 2 days (open bars, n=59) and 5-7 days (hatched bars, n=53) after exposure to the irritant. Results are expressed as mean±SEM using the Mann Whitney test for statistical evaluation of the difference between the groups.

Table 6: Statistical evaluation expressed as p values of differences in epidermal and dermal changes between 2 and 5-7 days of experiment.

Lesion	M	U	N	A	H	E	G
2 days vs. 5-7 days	P<0.0001	P<0.0001	P>0.05	P>0.05	P>0.05	P<0.0001	P>0.05

Lesion	IA	IS	dH	dN	F
2 days vs. 5-7 days	P<0.0014	P<0.0001	P>0.05	p<0.0001	P<0.0001

Fig. 7: Iodine treatment 45 min after irritant application: comparison between 2 days and 5-7 days experiment.



Twelve parameters were determined: epidermal microvesicles (M), epidermal ulceration (U), epidermal necrosis (N), epidermal acanthosis (A), epidermal hyperkeratosis (H), epidermal encrustation (E), grade of area of epidermal acanthosis (G), dermal inflammation acute (IA), dermal inflammation subacute (IS), dermal hemorrhage (dH), dermal necrosis (dN), dermal fibrosis (F). Animals were sacrificed 2 days (open bars, n=9) and 5-7 days (hatched bars, n=15) after exposure and treatment. Results are expressed as mean±SEM using the Mann Whitney test for statistical evaluation of the difference between the groups.

Table 7: Statistical evaluation expressed as p values of differences in epidermal and dermal changes between 2 and 5-7 days of experiment (45 min interval between exposure and iodine treatment).

Lesion	M	U	N	A	H	E	G
2 days vs. 5-7 days	P>0.05	P<0.01	P>0.05	P<0.001	P<0.05	P<0.0005	P<0.005

Lesion	IA	IS	dH	dN	F
2 days vs. 5-7 days	P<0.005	P<0.005	P>0.05	P<0.0005	P<0.0001 No calculation

Histopathological comments

The iodine acted as a dermo-protectant either by promoting a reparative reaction, expressed by epidermal proliferation (hyperplasia) originating from the undamaged margins of the lesion, or by slowdown and cessation of further deterioration of the SM-induced vescico-ulceratice process. No matter what is the mechanism of the drug protective effect, **findings of acanthosis and hyperkerathosis covering more extensive skin tested areas, are the most reliable histological proof for the effectiveness of the tested drug in the above mentioned experimental model**.

Analysis of the histopathological findings of the present experiment indicates that application of iodine 15 min following exposure to SM was more effective than 30 min interval between exposure and treatment, by promoting more effective healing process of the previously damaged skin (animals sacrificed 2 days after treatment) (Figures 1, 2, Tables 1, 2). The 15 min-interval of iodine application induced significant ($p<0.01$) acanthosis and hyperkerathosis, which had a significant extension (grade of area of epidermal acanthosis $p<0.001$). The significant reduction ($p<0.01$) of dermal necrosis, hemorrhage and acute inflammation at the 15 min interval, is an additional indication that the damaging effect of the SM was more effectively subsided using the shorter interval alternative. Nevertheless, in spite of the fact that, at the 30 min interval between exposure and treatment, the healing parameters were not elevated, there was significant reduction in epidermal microvesicles ($p<0.05$), ulceration ($p<0.05$) and encrustation ($p<0.01$). In addition, dermal acute inflammation ($p<0.001$), necrosis ($p<0.001$) and hemorrhage ($p<0.01$) were also significantly reduced at 30 min interval.

In the 45 and 60 min intervals between exposure and treatment (sacrifice 5-7 days after treatment) (Figures 3, 4, Tables 3, 4) relatively severe damage in the exposed skin was observed, namely, the iodine preparation was less effective than in the 15 and 30 min intervals. Nevertheless, the application of iodine 45 min following SM exposure was effective in reducing the epidermal microvesicles and ulceration ($p<0.05$), as well as significantly promoted acanthosis ($p<0.05$) and the epidermal hyperplasia on more extensive areas ($p<0.001$) and reduced dermal subacute inflammation ($p<0.01$). In addition, even at 60 min interval between exposure and iodine treatment, which showed the weakest protection, reduced epidermal microvesicles ($p<0.05$) and promotion of epidermal hyperplasia ($p<0.05$) on extensive area were observed.

The present study enables comparison of experiments in which the animals were sacrificed 2 days and 5-7 days after treatment (Figure 6, Table 6). In the longer experiment significantly increased damage to the epidermis (i.e, ulceration, microvescicle formation and encrustation) and to the dermis (acute and subacute inflammation, necrosis and fibrosis) was observed. At the same time, the grades of acanthosis and the areas covered by acanthosis became much more prominent, indicating that the grade of healing was also function of time passed from exposure until sacrifice.

When the skin samples of 2-days sacrifice of the different post-SM exposure intervals are compared, it is becoming evident that as long as the iodine is applied earlier it has better protective activity. It can be clearly demonstrated by the relatively high degree of acanthosis at 15 min interval between exposure and treatment (grade 2.4, Fig. 1), moderate (grade 1.2, Fig. 1) at 30 min interval and low (grade 0.5, Fig. 5 or 7) at 45

min interval. Similar phenomenon can be seen in the other regenerative parameters, i.e. hyperkerathosis and grade of area of epidermal acanthosis. Interestingly, 5-7 days after the treatment these factors increased in both SM only and in the 45 min interval.

POST EXPOSURE TREATMENT WITH IODINE PROTECTS AGAINST SULFUR MUSTARD-INDUCED SKIN LESIONS.
U Wormser[1], B Brodsky[1], A Sintov[2] and A Nyska[3]. [1]The Hebrew University, Jerusalem, Israel; [2]Ben Gurion University of the Negev, Beer-Sheva, Israel [3]National Institute of Environmental Health Sciences, NIH, NC, USA.
Sponsor: E Hoffer.

Sulfur mustard (SM) is a powerful vesicant employed as a chemical warfare. The currently available pharmacological antidotes against this blisterogen are of limited use. The present study demonstrates the protective effect of topical iodine preparation as post exposure treatment against SM (1.2mg net liquid) -induced lesions using the fur-covered guinea pig skin model. Iodine treatment 15 min after SM exposure resulted in statistically significant reduction of 48%, 50% and 55% in dermal acute inflammation, hemorrhage and necrosis, respectively, whereas the epidermal healing markers, hyperkerathosis and acanthosis were significantly elevated by 72% and 67%, respectively (skin analyzed 2 days after treatment). These healing parameters are the most reliable histological proof for the effectiveness of iodine. At longer interval of 30 min between SM exposure and iodine treatment there was significant degree of protection albeit to a lesser extent than that observed in the shorter interval. Although the epidermal healing markers were not elevated, the degenerative parameters such as epidermal microblisters and ulceration, and dermal acute inflammation, hemorrhage and necrosis were significantly reduced by 35%, 67%, 43%, 39% and 45%. At 45 min interval between exposure and treatment there was also certain degree of protection expressed by significant reduction in dermal acute inflammation and necrosis although the epidermal acanthosis and hyperkerathosis were reduced, indicating for low healing potential. At interval of 60 min between exposure and treatment, iodine was less efficient, nevertheless, significant reduction in epidermal microblisters and elevated acanthotic area were observed even at this long interval. Gross pathology analysis showed strong protective effect at intervals of 15 and 30 min and to a lesser extent at 45 and 60 min. The present findings suggest the iodine preparation as a potential antidote against skin lesions induced by SM (supported by USAMRMC Cooperative Agreement No. DAMD17-98-2-8009).